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(54) Title: NUCLEIC ACID DETECTION METHOD BY TRIPLE HELIX FORMATION

(57) Abstract

A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising (a) amplifying said target nucleic acid so that the product of the amplification reaction includes a purine rich region, (b) contacting the sample with a peptide nucleic acid able to bind to at least a portion of said target sequence; and (c) detecting the presence of triplex DNA structures. The detection is suitably effected directly, for example using a surface plasmon resonance detector.

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NUCLEIC ACID DETECTION METHOD BY TRIPLE HELIX FORMATION

The present invention relates to a method of detecting specific target DNA sequences, and in particular to the products of amplification reactions, as well as to reagents and apparatus used in that method.

Many methods are known in order to detect the presence of particular target DNA sequences in a sample. A substantial proportion of these methods require that the DNA is denatured to single stranded form and then this sequence is hybridised or otherwise allowed to bind to a labelled sequence specific probe.

The target sequences are frequently subjected to amplification reactions, for example the polymerase chain reaction or the ligase chain reaction, in order to increase the amount of the target sequence to detectable levels.

Other methods of detecting sequences include the use of intercalating dyes which are incorporated into the sequences during the amplification reaction. However such methods are relatively non specific as the dyes will intercalate with any amplification product, even if they are the result of non-specific amplification products.

Other assays such as the TAQMAN™ assay utilise complex probes which include reporter and quencher moieties during the course of the amplification process. These probes hybridise to single stranded target sequences during the amplification reaction and are then digested by the enzymes carrying out the reaction. The relationship between quencher and reporter molecule of the probe produces a signal which can be monitored. The probes used in this case however, are complex and expensive.

It is known that peptide nucleic acids will strand invade DNA at purine rich sites to form triplex structures (P.E. Nielson et al., Science, 1991, 254, p1497-1506, Turney D.Y. et al,. Proc. Natl. Acad. Sci. USA , 1993, 90, 1667-1670). The mechanism by which this is effected is illustrated diagrammatically hereinafter in Figure 1.

The applicants have found that this phenomenon can be used in detection of target DNA sequences.

A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising

(a) amplifying said target nucleic acid so that the product of the amplification reaction includes a purine rich region,

(b) contacting the sample with a peptide nucleic acid able to bind to at least a portion of said target sequence; and

(c) detecting the presence of triplex DNA structures.

The method enabling the direct detection of target sequences, for example amplification products without the usual denaturation step required for duplex formation with a nucleic acid probe.

The expression "purine rich region" means that the sequence is suitable for strand invasion by a peptide nucleic acid (PNA). Such regions suitably contain at least four consecutive purine residues.

The reaction in step (a) above is suitably effected in the presence of a buffer, and preferably a low salt buffer for example containing 50mM or less of salt as this favours triplex formation as compared to DNA:DNA duplexes. Furthermore, the pH of the buffer used will depend on the precise nature of the PNA

employed. If C's are used in the PNA strand to strand invade G's on duplex DNA, careful consideration has to be given to the pH of the buffer as the C involved in forming the Hoogsteen base-pair needs to be protonated, requiring a buffer of low pH, for example of less than 4.5.

The peptide nucleic acid used in the method of the invention may be single stranded or it may be bis-PNA. Preferably, the peptide nucleic acid used in the method is a bis-PNA as this results is a faster strand invasion process and a more stable triplex product.

Bis-PNA will comprise of two anti-parallel strands joined by a hydrophilic linker. One strand will be designed for Watson-Crick recognition of DNA within the target sequence, and the other strand is designed for Hoogsteen recognition of a PNA-DNA duplex. Such acids will be optimal for PNA_2DNA triplex stability and thus enhance strand-displacement binding to double-stranded DNA.

Peptide nucleic acids used will suitably contain a sequence of poly-T's or poly-C's.

The target nucleic acid is first subject to an amplification reaction such as the polymerase chain reaction (PCR) or ligase chain reaction (LCR), preferably PCR. The product may be exposed to the peptide nucleic acid during or after the amplification reaction, but is preferably exposed to the peptide nucleic acid after completion of the amplification reaction.

Where the target nucleotide sequence contains or is selected such that it contains a purine rich region, the method can be carried out directly. Where such regions do not exist in the target sequence, they may be introduced during the amplification

reaction. In this case, the amplification will be effected using one or more primers which comprise a plurality of pyrimidines, suitably at the 5' end thereof. This region will chain extend during the extension phase of the amplification (as illustrated in Figure 2 hereinafter). The 3'-end of both amplified strands of the amplification obtained using these primers should now contain the purine rich sites. Indeed, PCR products, that were tagged in this manner, have been cloned and sequenced and were found to have the poly-purine stretches incorporated at their 3'end. This ensures that a suitable PNA binding purine rich region is contained within the amplification product.

Primers of this sort form a further aspect of the invention.

The triplex formed may be detected using various methods in step (b). For example, gel retardation methods may be used. When the product is subjected to gel electrophoresis, for instance on a non-denaturing polyacrylamide gel, and then stained using conventional reagents such as ethidium bromide, the presence of a retarded triplex fraction can be observed.

This method however is relatively slow. Furthermore, comparison with a similar sequence which is not in the form of a triplex is required as a standard.

Preferably therefore, the detection is effected using a capture assay. The capture agent in this case is suitably the PNA sequence which is immobilised on a support. The sample is then contacted with the support whereupon any target sequence present will become associated with the PNA on the surface. It can then be detected using any of the known techniques.

In a particularly preferred embodiment, the support is a waveguide of a detection device which operates using evanescent wave detection. An example of such a device is a surface plasmon resonance detector. This allows the direct and rapid detection of target nucleotide sequence within a sample.

Thus a product of the amplification reaction is simply allowed to flow over the waveguide of such a detector and the presence of an amplicon can be detected in something approaching "real time".

In a further aspect, the invention provides a kit for use in the method of the invention. These kits suitably comprise a PNA designed to form a triplex with a target DNA. Optionally also, it may contain primers which can be used in the amplification of the target DNA, in particular primers which are 5'-tagged with pyrimidines.

The kit may also comprise a waveguide of a evanescent wave detector and particularly a surface plasmon resonance detector having supported thereon, the peptide nucleic acid which specifically binds a target DNA sequence.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 illustrates diagrammatically PNA:DNA triplex formation;

Figure 2 illustrates diagrammatically the incorporation of purine rich regions into an amplification product, using 5'-tagging of primers with polyramidine sequences; and

Figure 3 illustrates triplex formation on the surface of a surface plasmon resonance detector.

Example 1

Triplex Formation

The ability of PNA to form triplex structures with PCR products has been demonstrated using gel retardation studies. Two PCR products were chosen for study. One has a sequence capable of forming triplexes with a PNA probe i.e. contains poly-A sites.

PCR82

5'

ATAAATACAACCAACAAAATAAATAGTCATAAAATTGTATACATTAGCAATGCATACC ACAAAGTTCTAAGTACTAAAATAT 3' (SEQ ID NO 1)

The other does not contain poly-A sites and acts as a negative control.

PCR 175

5'

GCGAAACGGAACATAGCCCAAACCAAGAGGCTTGCCTCTTGGGGTTGTAGGACATTCT ATACGGAGTTACAAAGGAAGCAGGTAGACGAAGCGACCTGGAAAGGTCCGTCGTAGAGGGTAAC AACCCCGTAGTCGAAACTTCGTTCTCTCTTGAATGTATCCTGAGTACGGCGGAACACGTGAAA 3' (SEQ ID NO 2)

Two types of PNA probe were used, one was a linear sequence and contains a sequence of poly-T's

PNA057

N TTTTCCTTCCCTTTT C (SEQ ID NO 3)

The other, a bis-PNA of the same linear sequence but composed of two anti-parallel strands joined by a hydrophilic linker. One

strand was designed for Watson-Crick recognition of DNA and the other strand is designed for Hoogsteen recognition of a PNA-DNA duplex and should be optimal for PNA_2DNA triplex stability and thus enhance strand-displacement binding to double-stranded DNA.

PNA058

N TTTTCCCTTCCTTTT LLL TTTTCCTTCCCTTTT C (SEQ ID NO 4)

Each PCR product (5 µg/ml) was incubated with each PNA probe (10 µg/ml), at 37°C in 0.5 X TE buffer (1 mM Tris.HCl, 0.1 mM EDTA, 5 mM NaCl, pH 8.0) for varying time intervals before the reaction was terminated by adding 150 mM HBS, pH 7.4 on ice. Samples were run on a non-denaturing 12% polyacrylamide gel. The electrophoretic mobility of the triplex PNA_2DNA was compared to the duplex DNA of the relevant PCR product and visualised by EtBr staining. Triplex structures were observed suggesting that PNA can directly detect double-stranded PCR products.

The results of the gel retardation studies showed that single-stranded PNA did not strand invade the PCR products within the first 60 minutes. (This is backed up in the literature where it has been demonstrated that the association of a bis-PNA with a single strand of homopurine DNA gives a complex that is significantly more stable than the one formed with two single PNA strands due to a more favourable entropy of reaction.)

Bis-PNA, however, formed a triplex within the first 10 minutes of reaction.

Example 2

<u>Detection of triplexes on a surface plasmon resonance (SPR) surface.</u>

Biotin labelled bis-PNA (50 µg/ml) was linked to a dextran surface (Biacore, SAchip) via a streptavidin-biotin interaction. A sample of both PCR products (10 µg/ml), in water, was flowed

over this sensor surface and were detected by a change in refractive index. The SPR system could differentiate between purine-rich and non-purine rich PCR products in near real time (See Figure 3).

Claims

1. A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising

(a) amplifying said target nucleic acid so that the product of the amplification reaction includes a purine rich region,

(b) contacting the sample with a peptide nucleic acid able to bind to at least a portion of said target sequence; and

(c) detecting the presence of triplex DNA structures.

2. A method according to claim 1 wherein the peptide nucleic acid is bis-PNA.

3. A method according to claim 1 or claim 2 wherein the amplification product is exposed to the peptide nucleic acid during or after the amplification reaction.

4. A method according to claim 3 wherein the amplification product is exposed to the peptide nucleic acid after completion of the amplification reaction.

5. A method according to any one of the preceding claims wherein the amplification reaction is a polymerase chain reaction.

6. A method according to any one of the preceding claims wherein the target nucleic acid contains a purine rich region.

7. A method according to any one of the preceding claims wherein a purine rich region is introduced into the amplification product during the amplification reaction.

8. A method according to claim 7 wherein primers used in the amplification comprise a plurality of pyrimidines at the 5' end thereof.

9. A method according to any one of the preceding claims wherein the peptide nucleic acid is immobilised on a support.

10. A method according to claim 9 wherein the support is a waveguide of a detection device.

11. A method according to claim 10 wherein the detection device is a surface plasmon resonance detector.

12. A method according to any one of claims 1 to 8 wherein the triplex structure is detected by a gel retardation method.

13. A primer comprising a sequence which hybridises to an end region of a target nucleic acid sequence, and a plurality of pyrimidine residues at a 5'region thereof.

15. A kit for carrying out a method according to any one of the preceding claims, said kit comprising a peptide nucleic acid sequence which is specific for a target nucleotide sequence.

16. A kit according to claim 14 wherein the peptide nucleic acid is immobilised on a waveguide of an evanescent wave detector apparatus.

17. A kit according to claim 15 wherein the evanescent wave detector apparatus is a surface plasmon resonance detector.

18. A kit according to any one of claims 15 to 17 which further comprises a primer according to claim 13.

19. A method for detecting a nucleotide sequence according to claim 1, substantially as hereinbefore described.

Fig.1.

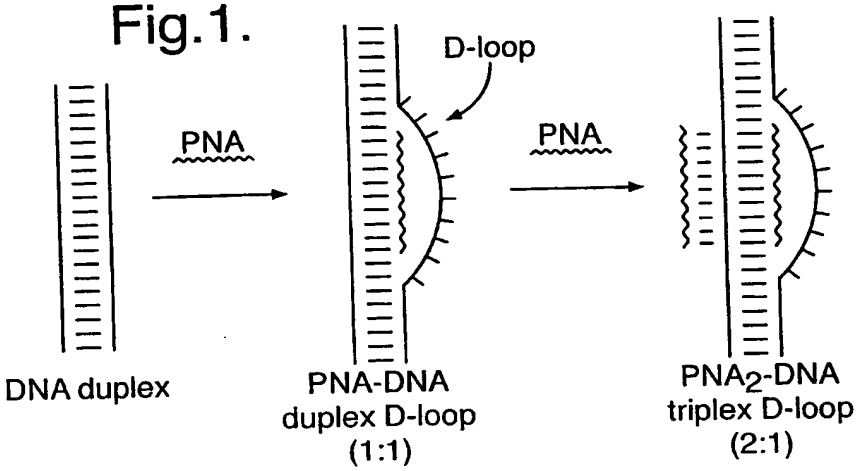

D-loop
PNA
PNA
DNA duplex
PNA-DNA
duplex D-loop
(1:1)
PNA2-DNA
triplex D-loop
(2:1)

Fig.3.

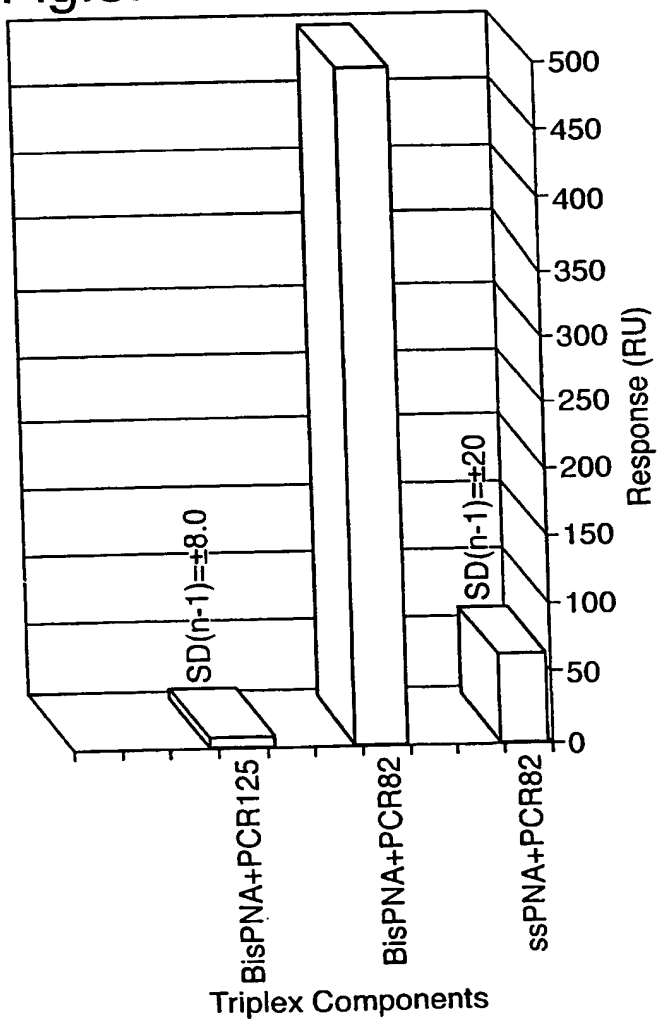
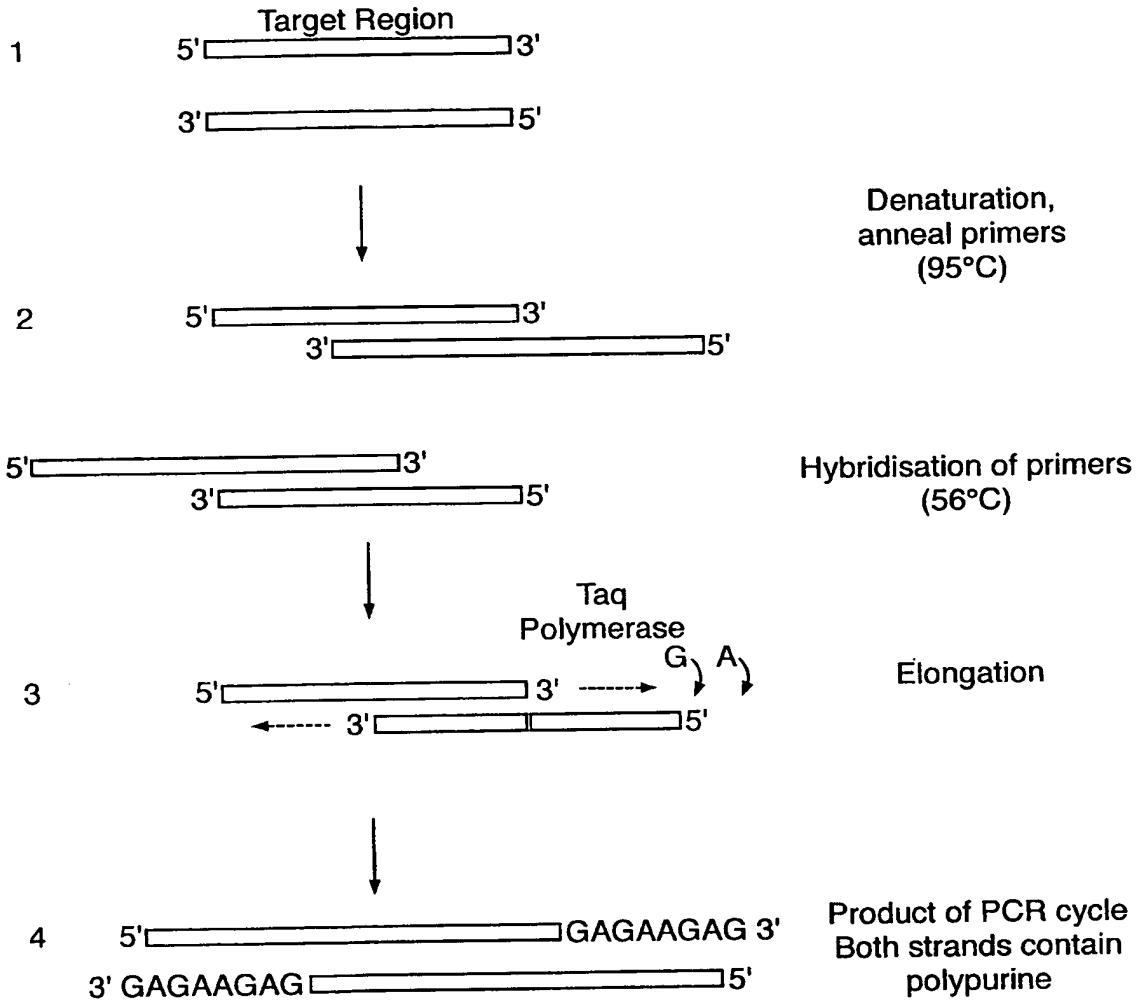

500
450
400
350
300
250
200
150
100
50
0
Response (RU)
SD(n-1)=±8.0
SD(n-1)=±20
BisPNA+PCR125
BisPNA+PCR82
ssPNA+PCR82
Triplex Components

Fig.2.


Target Region
1
5'
3'
3'
5'
Denaturation, anneal primers (95°C)
2
5'
3'
3'
5'
5'
3'
3'
5'
Hybridisation of primers (56°C)
Taq Polymerase
G
A
3
5'
3'
3'
5'
Elongation
4
5'
GAGAAGAG 3'
3' GAGAAGAG
5'
Product of PCR cycle Both strands contain polypurine
KEY:
=Complementary to target
=5'-polypyramidine tail (C,T)

INTERNATIONAL SEARCH REPORT

International Application No
PC./GB 99/02317

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 11390 A (IDEXX LAB INC) 9 July 1992 (1992-07-09) page 10, line 7 -page 11 page 34, paragraph 1; figure 1 ---	1-19
Y	WO 97 14793 A (UNIV BOSTON) 24 April 1997 (1997-04-24) page 5, line 11 - line 17 page 13 -page 14, paragraph 1 --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
4 November 1999	18/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040. Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate. of the relevant passages	Relevant to claim No.
Y	SEEGER C ET AL: "PNA-MEDIATED PURIFICATION OF PCR AMPLIFIABLE HUMAN GENOMIC DNA FROMWHOLE BLOOD" BIOTECHNIQUES, vol. 23, no. 3, 1 September 1997 (1997-09-01), pages 512-514, 516/517, XP000703351 ISSN: 0736-6205 the whole document	1-19
Y	J WANG ET AL: "Peptide nucleic acids probes for sequence-specific DNA biosensors" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 118, no. 33, 21 August 1996 (1996-08-21), pages 7667-7670, XP002094817 ISSN: 0002-7863 the whole document	10,11, 16,17
Y	KAI E ET AL: "Novel DNA detection system of flow injection analysis (2): The distinctive properties of a novel system employing PNA (peptide nucleic acid) as a probe for specific DNA detection" NUCLEIC ACIDS SYMPOSIUM SERIES, vol. 37, 1997, pages 321-2, XP002121513 the whole document	10,11, 16,17

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9211390 A	09-07-1992	EP 0566670 A	27-10-1993
		US 5800984 A	01-09-1998
WO 9714793 A	24-04-1997	AU 7016096 A	07-05-1997